Human transcriptome response to immunization with live-attenuated Venezuelan equine encephalitis virus vaccine (TC-83): Analysis of whole blood

Running Title: Human Immune Responses to Vaccination with Live-Attenuated VEEV

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Abstract:

Venezuelan equine encephalitis virus (VEEV) is an important human and animal alphavirus pathogen transmitted by mosquitoes. The virus is endemic in Central and South America, but has also caused equine outbreaks in southwestern areas of the United States. In an effort to better understand the molecular mechanisms of the development of immunity to this important pathogen, we performed whole genome transcriptional analysis from whole, unfractionated human blood of patients who had been immunized with the live-attenuated vaccine strain of VEEV, TC-83. We compared changes to the transcriptome between naïve individuals who were sham vaccinated with saline to responses of individuals who received TC-83. Significant transcriptional changes were noted at days 2, 7, and 14 post vaccination. The top canonical pathways revealed at early and intermediate time points (days 2 and 7) included the involvement of the classic interferon response, interferon-response factors, activation of pattern recognition receptors, and engagement of the inflammasome. By day 14, the top canonical pathways included oxidative phosphorylation, the protein ubiquitination pathway, natural killer cell signaling, and B-cell development. Biomarkers were identified that differentiate between vaccinees and control subjects, at early, intermediate, and late stages of the development of immunity as well as markers which were common to all three stages but distinct from the sham-vaccinated control subjects. The study represents a novel examination of molecular processes that lead to the development of immunity against VEEV in humans and which may be of value as diagnostic targets, to enhance modern vaccine design, or molecular correlates of protection.

Introduction:

Venezuelan equine encephalitis virus (VEEV) is a single-stranded, positive sense RNA virus and a member of the *Alphavirus* genus of the family *Togaviridae*. Among the New World alphaviruses, VEEV is considered to be one of the most pathogenic for humans.[1] The Trinidad strain of VEEV was originally isolated from the brain of an infected donkey in 1938 and is classified as subtype I A/B.[2, 3] In total, there are six antigenic varieties or subtypes of VEEV; however only subtypes I A/B and IC have been associated with epizootic outbreaks.[3] Vertebrates, especially equids, can serve as both recipients of viral infection and amplification reservoir hosts. Transmission of the virus typically occurs from the bite of an infected mosquito. The infectious course of epizootic strains in equines is characterized by high-titer viremia, which contributes to the disease state as well as the typical transmission cycle, as the infected animal or human is again fed upon by mosquitoes.[4]

VEEV is classified as a Category B biological threat agent by the Centers for Disease Control (CDC) and has reportedly been developed as a biological weapon in the past.[4, 5] The virus is highly infectious by the aerosol or inhalational route, and incidental infection has been problematic to laboratory personnel due to accidental exposures.[6, 7] Typical disease cases present with flu-like symptoms, including fever, chills, headache, and malaise.[6] Encephalitis occurs in a small percentage of cases, and most often in children; additional symptoms of severe disease include severe headache, photophobia, ataxia, disorientation, and convulsions.[3] Diagnosis of disease is achieved primarily through direct detection, either by virus isolation from a biological sample, detection of antigen by enzyme-linked immunosorbent assay (ELISA) or nucleic acid by polymerase chain reaction, or indirectly by detection of IgM by ELISA. For isolates or IgM

positive samples, the plaque reduction neutralization test (PRNT) is particularly useful for distinguishing VEEV infection from other related alphavirus infections.[3, 8]

Currently, there is no FDA-approved vaccine or therapeutic available for the prevention or treatment of Venezuelan equine encephalitis (VEE). However, there are two investigational new drug (IND) vaccines that are available for at-risk laboratory personnel.[8] The first vaccine, TC-83, is a live-attenuated virus developed in 1961 by serial passage of the virulent Trinidad strain of VEEV though tissue culture in fetal guinea pig heart cells.[9] The second, C-84, is a formalin-inactivated version of the TC-83 strain.[10]

Live-attenuated TC-83 has been used extensively in humans and has demonstrated high protective data as measured by the production of neutralizing antibodies; however, the rate of nonresponders is approximately 20-25%, as measured by the failure to produce neutralizing antibodies against VEEV following immunization.[8] In addition to naturally-occurring nonresponse, there have been demonstrations of immune interference contributing to the lack of neutralizing antibody production for individuals receiving sequential alphavirus immunizations, including circumstances when individuals received eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV), or Chikungunya virus (CHIK) prior to immunization with VEEV.[8, 11]

In recent years, the reemergence of VEEV has prompted public health concern and highlighted the persistent need to develop modern vaccines which can achieve FDA-approval or to develop effective therapeutics which can be licensed. Additionally, high rates of primary vaccine failure as well as evidence of sexually dimorphic responses to vaccination are compelling reasons that there is a current need to develop modern, rational vaccines against VEEV.

There are few studies to date which have been conducted to assess the molecular responses to VEEV. Host transcriptional responses to VEEV have been reported in a small number of animal model systems (mice and cynomolgus macaques) and in one *in vitro* study of human PBMC cells.[12-18]

VEEV infection has been noted to stimulate the involvement of genes relating to inflammation and immune processes in mice, nonhuman primates, and in human PBMC cells.[13, 14, 18] Transcripts demonstrating increased expression in mouse brain were predominantly chemokine genes (CXCL9, CXCL10, CXCL11, CXCL13, CCL3, CCL5, and CCL12) which presented at an intermediate timeframe of viral infection (days 3 and 4 post infection).[14] The timing of expression of chemokine genes coincided with the first biphasic peak in permeability of the blood-brain barrier (BBB) at day 3 post infection with virulent VEEV, with inflammation in the brain denoted by vessel thickening, endothelial cuffing, and infiltration of neutrophils into the brain.[14, 19] Similar induction of chemokine transcripts was noted in nonhuman primates by Koterski et al.[13] However, Koterski et al. noted increased expression of inflammatory response genes (including CXCL11, CCL3, as well as IL1RN, IRF7, and TNFAIP6) more notably in the spleen than in the brain, as it has been observed in mice.[13] Hammamieh et al.[12] described transcriptional profiles of PBMCs using the same nonhuman primates reported by Koterski and colleagues[13] and noted increased transcription of both CCL13 and CCL18 chemokine genes. Induction of chemokine transcripts in human PBMCs infected with the live-attenuated strain of VEEV (TC-83) was noted *in vitro* by the increased expression of CXCL11, CCL3, CCL5, CCL7, and CCRL2 in both naïve and responder PBMC samples from human volunteers who were either VEEV vaccine-naïve or had previously presented titers in response to VEEV vaccination.[18]

Both type I and type II interferon responses to viral infection were observed across multiple tissue types and species, with increased transcript expression noted for IFNB1, IFNG, IRF7, several forms of OAS transcripts, MX1, MX2, and STAT1.[13-18] Other notable patterns of transcript expression that have been previously reported include widespread engagement of signaling moieties that are key players in pattern recognition receptor (PRR) detection of bacteria and viruses, including such transcripts as IL6, DDX58, TLR3, TLR7, and CASP1.[18]

The purpose of the present study is to examine the molecular changes that occur in humans in response to VEEV immunization with the overarching goal to provide an in-depth analysis of the molecular events which contribute to the development of immunity, and hence may inform any attempts to design a more effective vaccine or therapeutic. Furthermore, there are significant gaps in the foundation of knowledge surrounding the host cell signaling pathways required to combat viral infection and propagation.[7] To that end, we have conducted a whole transcriptome analysis of human genes which are modulated in response to VEEV immunization; samples were derived from whole, unfractionated blood at various time points, both before and after immunization, and were compared with sex- and age-matched control samples at each time point.

Results:

Overall effects of immunization with TC-83 over time

Comparison of global gene expression values across time (i.e., at 1, 4, 8 hrs and at days 1, 2, 7, 14, 21 and 28 post vaccination), in response to treatment, and as a function of both time and treatment concomitantly, yielded results that met statistical significance criteria (cut-off p-value) at days 2, 7, and 14 post vaccination when measured against time-matched control samples (Table 1). The false discovery rate was set to the limit of 10% using the Step-up multiple test correction method. At day 2 post vaccination, data analysis revealed 3,511 differentially expressed transcripts. There were 424 differentially expressed transcripts detected at day 7 and 21,343 transcripts at day 14. In comparison with samples from mock-vaccinated individuals, there were no statistically significant changes in gene expression levels for TC-83 vaccinated individuals at any other time point. Data were then further constrained by examining the fold change of gene expression of each transcript; only transcripts with a fold change of ≥ 2 were included in further analyses (Figure 1). Applying these criteria reduced the total number of significantly expressed transcripts to 1,142 covering days 2, 7, and 14 post vaccination.

Alteration of transcript expression at specific times included thousands of different genes, with surprisingly little overlap. The first time point where a difference in gene expression in the TC-83 vaccinated individuals could be detected, relative to sham-vaccinated control subjects, was at day 2 (Figure 1). Of the 225 transcripts that were differentially expressed on day 2 with at least a 2-fold change in expression, only 32 overlapped with transcripts at both days 7 and 14. On day 7, we detected 14 differentially expressed transcripts unique to day 7; while, on day 14 post vaccination, 756 transcripts were detected and unique to this time point. The graphical interactions displayed by Venn diagramming show a clear distinction in gene expression across

time; from these results, the patterns of gene expression were stratified based on early (day 2), intermediate (day 7), and late (day 14) response to vaccination (Figure 1).

The variation induced in transcript expression between treatment groups and samples was assessed by Principal Component Analysis (PCA) (Figure 2). The primary dimension of the PCA analysis, reflecting the greatest variation in gene expression, was attributed to the effect generated by vaccination with TC-83 (treatment, depicted as grouping by color), accounting for 23.8 % of transcript expression variation. The second dimension of variation in transcript expression was due to changes over time following vaccination (depicted as increasing size of spheres) and accounted for 7.5 % of the variation observed. Finally, the third dimension of variation can be explained by the changes that occurred as a factor of the interaction of time and treatment together, which accounted for 3.5% of the total variation.

Cellular pathway analysis for changes in the transcriptome induced by immunization with TC-83 in humans

We conducted pathway analysis using Ingenuity Pathway Analysis software (Ingenuity, Redwood City, CA) to better understand the scope and function of the molecular responses generated in humans in response to TC-83 vaccination. The observed host responses covered a variety of pathways involved in disease processes, molecular and cellular functions, and physiology system development and function (Table 2). On day 2 post vaccination, representing the early transcriptional response, there was noted involvement of specific transcripts which were indicative of a strong antimicrobial and inflammatory response, as well as transcripts that were characteristic of infectious disease, infection mechanisms, and organismal injury (Table 2). The molecular functions related to transcripts which were differentially expressed on day 2

represented cellular movement and development, cellular signaling, post-translational modification, and protein folding. These molecular functions routinely participate in the systemic organization of hematological function, immune cell trafficking, tissue development, muscular-skeletal development, and hematopoiesis. The most prominent signaling pathways induced upon VEEV vaccination included the interferon signaling pathway, activation of interferon-response factors by cytosolic pattern recognition receptors, involvement of pattern recognition receptors in the recognition of viruses and bacteria, the RIG1-like receptors as part of a classical innate antiviral immune response (i.e., the inflammasome), and the IL-6 signaling pathway. The responses observed at day 7 post vaccination were similar to those seen at day 2 with regard to a clear induction of infectious, inflammatory, and antimicrobial responses. In addition to induction of molecular and cellular functions (e.g., post-translational modification), cellular development and protein folding functions were also observed at day 2. In contrast, by day 7 post vaccination, molecular functions expanded to transcripts related to lipid metabolism and molecular transport. Similarly, overlapping physiological system functions relating to hematological development, hematopoiesis, immune cell trafficking, and muscular-skeletal development continued to be top factors through day 7 post vaccination. However, an evolving response was evident by the induction of transcripts involved with endocrine system development and function. Likewise, the top canonical pathways that were observed on day 7 post vaccination were predominantly similar to those seen at day 2 (i.e., interferon signaling, pattern recognition receptor activation of interferon-response factors, inflammasome-related transcripts) but also included transcripts which were involved in the pathogenesis of multiple sclerosis. Two parameters characterize a dramatic shift in the results from day 14 post vaccination: First, the molecular and cellular processes observed primarily involved those of nucleic acid metabolism, cell to cell signaling,

cellular compromise, gene expression, and molecular transport; and secondly, the top canonical pathways shifted from a strong interferon-driven response to one characterized by oxidative phosphorylation transcripts, protein ubiquitination, RAN signaling, T cell receptor signaling, and regulation of eIF4 and p70S6K signaling (Table 2).

The involvement of specific canonical pathways in this temporal study of transcriptional expression allowed us to compare and describe three distinct phases of human VEEV infection *in vivo*. We employed Ingenuity Pathway Analysis (IPA) to describe the involvement of individual transcripts and canonical pathways in the development of immunity following TC-83 immunization. During the earliest phase (day 2 post vaccination) there was a strong induction of interferon signaling genes and subsequent interferon-related factors (Table 3). Some of the most notable transcripts representing interferon signaling included IFIT1, IFIT3, MX1, OAS1, and IFI34, and many of these transcripts continued to display increased expression through day 7 as well. However, by day 14 most interferon signaling transcripts had returned to baseline levels (Table 3). Similarly, activation of interferon related factors was evident by day 2, including genes comprising the inflammasome (RIG1, also known as DDX58; MDA5, also known as IFIH1; LGP2, also known as DHX58; and a novel DEXD/H box helicase, DDX60), (Table 3). Increased transcription of genes involved in IL-6 signal transduction was highest at day 2 (e.g., IL1RN, SOCS1, and TNFAIP6) with noted decrease in IL-8 transcription (Table 3). Key signaling components of the JAK/STAT pathway were also noted to have increased transcription at day 2 following immunization which was sustained through day 7, but returned to baseline levels by day 14 (e.g., SOCS1, STAT1, and STAT2) (Table 3).

In sharp contrast, the canonical pathways that are highly represented by transcripts with increased expression by day 14 post immunization include that of oxidative phosphorylation

(e.g., COX7A2, COX16, UQCRB, UQCRH, PPA1), the protein ubiquitination pathway (e.g., UBR1, USP1, PSMA3, PSMC6, BIRC2, BIRC3, HSP90AA1), the ERK5 Signaling pathway (e.g., IL6ST, NRAS, RRAS2, ATF2), the Natural Killer Cell Signaling pathway (e.g., KLRC2, FYN, PRKC1, KLRK1, KLRC3, RRAS2, NRAS), and the B-Cell Development pathway (e.g., IL7R, IGKC, IGL@, IGHM) (Table 3).

Biomarker analysis and identification using Ingenuity Pathway Analysis

The temporal transcriptional responses from TC-83 vaccinated subjects and unvaccinated control subjects were evaluated with IPA to establish biomarkers following immunization; we employed analysis filters to enrich for biomarkers previously identified in biological fluids (e.g., blood, sera, plasma, and urine). The results were clustered into groups representing early, intermediate, and late biomarkers (Table 4) categories which distinguished biomarkers that were either unique to each stage of immune development or were common across all days following immunization. The biomarkers displayed in Table 4 were selected by restricting the analysis to the top 10 transcripts showing the greatest levels of change in expression, as well as showing consistent expression profiles over time, and for all potential probe sets which correspond to each transcript.

HLA phenotype and post vaccination titer

All study subjects were assessed for the development of neutralizing antibodies against live attenuated TC-83 at 28 days post vaccination; production of neutralizing antibody in response to vaccination is currently the gold standard measure of an immunity correlate of protection and denotes successful primary vaccination. Results of neutralizing antibody

production were compared with HLA phenotype to describe the potential contribution of MHC haplotype to the immunological response induced by the vaccine (Table 5). Control study subjects receiving only a saline injection were also included in this portion of the study to demonstrate the lack of antibody response as a result of mock vaccination. Table 5 displays a subset of MHC Class II haplotypes (i.e., DRB1 and DQB1). A single volunteer (Vaccinee 1) who displayed the HLA DQB1*0301 allele is included in the table; however the gene expression data from that individual was removed from the microarray data analysis due to primary vaccine failure. We noted that two of three "low" responders (Day 28 post vaccination titer < 100) displayed a shared HLA haplotype (DQB1 *0302). The DQB1 *0302 phenotype was also present in one of the "high" vaccine responders (Day 28 post vaccination titer >100). Complete HLA phenotype data for study subjects, including all MHC Class I and Class II haplotypes, may be requested from the corresponding author.

To address the potential role or contribution of certain HLA DQB1 alleles to vaccine outcome, a second ANOVA was performed to include neutralizing titer as a variable (i.e., low titer <100, high titer >100) (Supplemental Data Table 1). Temporal gene expression values in low and high titer immune response groups were compared to describe changes which could be observed between these two groups (Supplemental Data Table 2). While the expression of many genes met the criteria of statistical significance for differential expression, none of the significant genes met the cut off of 2-fold or higher change in expression level used in the primary analysis, suggesting that the pathways and processes that are critical for vaccine success or failure in humans are tightly regulated and may be influenced even by small changes in transcriptional expression.

Discussion:

VEEV is a reemerging pathogen with potential risk as both a public health and biological threat.[25] VEEV is classified as a category B biological threat agent by the US Centers for Disease Control and Prevention (CDC). The human disease caused by VEEV infection is difficult to assess as symptoms are clinically similar to other, more commonly occurring diseases, such as Dengue Fever.[20] Confirmatory diagnosis of VEEV requires specialized laboratory tests that are frequently unavailable in countries with limited medical and public health resources.[26] Small outbreaks of epizootic VEEV have been detected in endemic countries for decades; however, recent surveillance data covering Latin American nations (particularly Mexico, Panama, and Peru) have suggested that the annual number of VEEV cases have been seriously underrepresented, in large part because of misdiagnoses of infection with VEEV as Dengue Fever. Such misdiagnoses may comprise between 0.1 to 7% of all Dengue Fever infections.[26] As such, VEEV infection remains an important public health threat.

The advent and widespread application of vaccines has been hailed as one of the most profound achievements for public health in the 20th century. Successful vaccination is ideally mediated through both B and T cell mediated responses. Vaccine responses, as correlates of protection, are often measured by the ability of the vaccine to generate measurable levels of neutralizing antibodies and are usually the only correlate of protection data available in vaccination studies.[27]

Currently, there is no FDA-approved vaccine for human immunization against VEEV, although there is an Investigational New Drug (IND) vaccine, live-attenuated VEEV TC-83, which has been used for decades by military and at-risk laboratory personnel.[8, 21] The mechanism of protection induced by vaccination with TC-83 is believed to be through the production of

neutralizing antibody, but other molecular mechanisms of protection are not well understood or defined.[8, 21] The present study explored the sequential molecular events, *in vivo,* which occur following human immunization with TC-83, and which lead to the development of immunity.

The early and sustained engagement of interferon signals and interferon response factors beginning on day 2 and extending to day 7 observed post vaccination are indicative of a traditional innate antiviral immune response. There is an extensive overlap between the molecules that exhibit changes in transcript expression and the canonical pathways in which they participate, particularly between genes of the interferon response, interferon-response factors, activation of pattern recognition receptors, and engagement of the inflammasome. Potent induction of expression in IFIT1 (ISG54), IFIT3, IRF7, TLR7, and OAS 1-3 represent induction of a classic type I interferon signaling mediated in response to single-stranded RNA viruses.[22-25] IFIT1 has been shown to act as a molecular receptor for 5' tri-phosphorylated RNA and consequently inhibit viral replication.[24] We also observed increased transcription of IFIT3 which contributes to antiviral signaling by bridging mitochondrial antiviral signaling and TBK1.[26]

Early induction of the broad-spectrum innate inflammasome response was noted as a consequence of immunization, spanning days 2 through 7. Engagement of the inflammasome has been shown to be classically mediated through TLR7, DDX58 (also known as RIG-1), IFIH1 (also known as MDA5), and DHX58 (also known as LGP2).[27] We found that TC-83 immunization caused transcriptional induction of DDX60, an RNA helicase related to DDX58 which has also been demonstrated in functional genomics studies to be required for RIG-1 or MDA5-dependent signaling in response to viral infection.[28-29] Satoh et al.[30] describe the importance of DHX58 (LGP2), an ATP-dependent RNA helicase, as a key modulator of both RIG-1 and MDA5-mediated responses ostensibly through activity which makes viral RNA more

accessible to either RIG-1 or MDA5 directly or by altering the cellular location of viral ribonucleoprotein complexes for greater access. Other proteins that can initiate anti-viral responses include IFIT2 (ISG56), RSAD2 (viperin), and ISG15; our results demonstrate strongly increased transcription for each of these transcripts on both days 2 and 7 following vaccination suggesting that the type I interferon response is primarily regulated through IRF3 activation.[31] Over expression of RSAD2 has been linked to expression and regulation by histone deacetylase 1 (HDAC1) which results in transcriptional repression; during VEEV-induced early engagement of the inflammasome, HDAC1 expression was not altered. Indeed, HDAC1 expression was not altered significantly until day 14 post vaccination.[32] Regulation of HDAC1 has been shown to be dependent both on the cell type and influenced by the physiological environment.[32]

The HLA-DQB1 phenotype has previously been associated with autoimmune disorders and suggested to be involved with hyporesponsiveness to vaccination.[18, 33-35] A number of studies also suggest that certain combinations of the DQB1 allele play an important role in linkage disequilibrium patterns.[36-38] From the current study, nine of ten vaccinated volunteers produced an effective immune response, as measured by the production of neutralizing antibody against VEEV. However, no trend in either HLA-DRB1 or HLA-DQB1 phenotype could be definitively determined with respect to linking the phenotype allele to an immunization outcome. We previously reported results of an *in vitro* assessment of changes in transcription in PBMCs from volunteers previously vaccinated with VEEV TC-83 in which it was suggested that there may be an inverse association between HLA DQB1 alleles and production of neutralizing titer.[18] In that instance, either the HLA DQB1 *0301 or *0302 allele was present in the samples of volunteers with the lowest neutralizing antibody titer. Interestingly, specific alleles of the HLA DQB1 haplotypes, including DQB1*0201 and DQB1*0302, have been reported to confer up to 50% of

the risk of heritable Type I diabetes.[38] We noted decreased transcription of several genes related to insulin signaling, IRS2, SGK, and IGF1R, during the course of vaccination and immune development, suggesting that the insulin signaling pathway may be involved in early responses to vaccination. Additionally, within the DRB1 haplotype, the DRB1 *1501 allele has been associated with Multiple Sclerosis.[39] The data suggest that the association between vaccine failure (i.e., vaccine nonresponders) and responders with low neutralizing titer may not necessarily be due to a random association with DQB1 *0301 or *0302 alleles, but rather these results prompt further study to test the hypothesis that primary vaccine failure and weak vaccine take can be explained, at least in part, by association with specific HLA haplotypes. Indeed, the answer to such questions may not ultimately rest on only one haplotype (e.g., DQB1) but may be influenced by the combination of specific DRB1 and DQB1 alleles. While the results are intriguing, it is clear that there are additional factors that affect both disease outcome and vaccination success; further work will need to be conducted to address the questions that such results inspire and with greater numbers of subjects to achieve statistical significance.

We queried the IPA analysis to evaluate the effects of vaccination on the microRNA population; changes in the expression of certain microRNA may represent an avenue of future investigation to suggest regulatory mechanisms for differentially expressed genes. Several microRNA factors were identified as having been effected by VEEV infection, including let-7, miR-21, miR30, miR-101, and miR-214; the presence of these microRNA suggest that the regulation of transcription of certain genes may also be influenced by microRNA. Further studies are needed to pinpoint the hypothesized involvement of specific roles these microRNA and what role each factor may play in the transcriptional regulation of genes and the timing of interaction

(transcription, translation, or post-translational modification of genes) (Supplemental Data Table 3).

The study is not without limitations. We were able to detect statistically significant changes in gene expression at days 2, 7, and 14, but at no other time points in the study. This could have been in part due to the restrictive statistical parameters used (i.e., 95% power, 0.001 two-sided t-test, 2-fold change filter, 0.5 CV). Future studies should include a time point between day 7 and day 14 to bridge the changes associated with a largely interferon-driven response and the beginning of development of immunity. Follow on studies may benefit from using a larger sample size to detect more discreet changes of gene expression, and potentially determine whether a correlation between HLA DRB1 or DQB1 alleles and neutralizing antibody production could be established. This would provide further support of previously published *in vitro* data.[18] The present study also utilized only male volunteers between the ages of 23-48 as a strategy to control confounding factors such as age and female sex hormone signaling; future studies should address potential differences in immune response due to age and gender, as well.

The changes observed from whole blood sampling of the transcriptome of subjects vaccinated with live-attenuated VEEV TC-83 provide the first glimpse of the molecular epidemiology events that contribute to the specific development of alphaviral immunity in a human host. The most profound changes were noted at days 2, 7, and 14 post vaccination and represent early, intermediate, and late transcriptional events. By day 14, it is not surprising that many of the top molecules which are differentially expressed are related to immunoglobulin genes (Table 2, Table 3, and Table 4). While the early and intermediate phases are dominated by interferon responses, driving innate anti-viral host responses, the events that occur at day 14 are among the most interesting and are represented by changes relating to oxidative phosphorylation,

protein ubiquitination, MAPK-related cell signaling pathways, and both natural killer signaling and B-cell development. These changes are similar to reports of involvement of ubiquitination in other alphaviruses. Indeed, nsP2 proteins of Sindbis, Semliki Forest, and Chikungunya viruses have been shown to inhibit cellular transcription by ubiquitination of Rpb1, a catalytic subunit of the RNAPII complex, suggesting a possible mechanism utilized by Old World alphaviruses to subvert the cellular antiviral response.[40] Differentially expressed transcripts for the MAPK pathway and for the pore-forming protein perforin and the family of granzymes have been suggested as a potential antiviral role in cytotoxic T lymphocyte (CTL) and natural killer (NK) cells in another positive sense RNA virus, the Japanese encephalitis virus infection.[41] The exploitation of similar mechanisms by VEEV, as suggested by our results, may represent highly conserved responses.

Biomarkers which are unique to each phase or common across all stages of infection have been identified with the potential to serve as a molecular signature of infection or as molecular correlates of protection. The HLA phenotype data combined with analysis of the immunity process in humans to VEEV vaccination establish new frontiers for further evaluation of identified HLA phenotypes and induced host genes for their contribution to genomic instability of certain phenotypes and production of neutralizing antibody titers, which are currently the gold-standard in terms of correlates of immunity.[18, 42] Additionally, the suggested host mechanisms affected by vaccination with live-attenuated VEEV TC-83 in humans revealed potential viral subversion strategies to achieve productive infection, which could be manipulated therapeutically or in immunization intervention protocols to achieve full protection against VEEV and related alphaviruses.

Patients and Methods:

Selection of volunteers:

The research protocol was conducted under Good Clinical Practice (GCP) quality standards, approved by the USAMRIID Institutional Review Board (IRB), and volunteers signed a written informed consent document (ICD) prior to enrollment in the study which described the purpose of the study, as well as the manner in which samples would be collected, used, and disposed. The study consisted of twenty male volunteers between the ages of 23 and 48 years. Male volunteers were selected for the study to reduce the confounding impact of hormonal variation on global gene expression. Additionally, each vaccinee was age-matched to a control volunteer. Study participants were individuals who had not previously received any alphavirus IND vaccines (i.e., against WEEV, EEEV, or VEEV). Prior to enrollment and participation in the study, all study participants were screened for antibodies by ELISA and PRNT[18] for prior exposure to new world Alphaviruses (VEEV, EEEV, and WEEV) and demonstrated to be negative for previous exposure. Participants were also genotyped for Human Leukocyte Antigen (HLA) allele expression, as previously described.[18] The *in vivo* study, conducted under Good Clinical Practice quality standards and approved human use protocol FY06-17, included ten vaccinees who received 0.5 ml of live-attenuated TC-83 VEEV (NDBR-102 vaccine) [roughly equivalent to 1.7×10^5 plaque forming units (PFU) of the virus] administered subcutaneously (SC) in the upper outer aspect of the arm, as well as ten control subjects who were administered 0.5 ml saline via the same procedure. Whole, unfractionated blood was collected at specific time points immediately prior to (0 h) and following vaccination (1, 4, 8 h and days 1, 2, 7, 14, 21, and 28). On day 56 post vaccination, serum was drawn from volunteers to assess development of neutralizing antibody titer against VEEV. The dataset is comprised of expressed transcripts from

9 responder vaccinees and 10 control subjects; one vaccinated subject was removed due to primary vaccine immunization failure. Total blood RNA samples from these individuals were subjected to microarray analysis.

RNA Isolation and sample preparation for microarray analysis:

RNA was isolated from whole, unfractionated blood using the PAXgene Blood RNA kit according to manufacturer's instructions (Qiagen, Valencia, CA). Briefly, RNA from whole blood was collected in PAXgene Blood RNA tubes from each volunteer at each time point. Samples were subjected to quality and concentration analysis using the Agilent RNA 6000 Nano BioAnalyzer kit, according to manufacturer's instructions (Agilent, Santa Clara, CA). Total RNA samples were then prepared for hybridization to the Affymetrix Human Genome U133 plus 2.0 Gene chip arrays according to manufacturer's specifications (Affymetrix, Inc., Santa Clara, CA). The microarray hybridizations were performed at the Core Laboratory Facility at the Virginia Bioinformatics Institute (Blacksburg, VA).

Microarray Data Analysis:

The gene expression data (Affymetrix .CEL files) were imported into Partek Genomics Suite v6.0 software (Partek Inc., St. Louis, MO). Using the Robust Multi-array Average (RMA) algorithm,[43] the gene expression data (Affymetrix gene probe sets) were normalized and $\log_2$ transformed. To detect differential expression, a 4-way ANOVA was constructed by using the restricted maximum likelihood (REML) approach to produce an unbiased estimate of variance.[44] The following equation describes the partitioning of time, vaccine type, and subject variability from variability due to biological and experimental noise:

Equation 1: $Y_{ijklm} = \mu + \text{Scan Datei} + T_j + V_k + S(V)_{kl} + T * V_{jk} + \varepsilon_{ijklm}$

Where Y_{ijklm} represents the m^{th} observation on the i^{th} Scan Date, j^{th} Time Point, k^{th} Treatment, l^{th} Subject. The common effect for the whole experiment is represented by μ, and ε_{ijklm} represents the random error present in the m^{th} observation on the i^{th} Scan Date, j^{th} Time Point, k^{th} Treatment, l^{th} Subject. The errors ε_{ijklm} are assumed to be normally and independently distributed with mean 0 and standard deviation δ for all measurements. The symbols T, *V*, *VT*, and *S(V)* represent effects due to time, vaccination type, treatment-by-time interaction, and subject-nested-within-treatment, respectively. Vaccine type and time are fixed effects; scan date and subject are random effects. Using this ANOVA model, gene expression data from 9 individuals from the VEE vaccine group were contrasted against those from 10 individuals of the placebo vaccination group (control group). The p-value for each condition was then corrected using the step-up false discovery rate (FDR) multiple test correction with a cut-off value of 0.1 to produce the list of significantly modulated genes (Table 1).[45] Contrasts between vaccinated and control subjects at each time point were achieved using Fisher's Least Significant Difference (LSD) of Log_2 transformed data and applying a further restriction of at least 2-fold change in gene expression (either up or down).[46] Requests for the complete microarray data should be directed to the corresponding author.

Ingenuity Pathway Analysis:

For the cellular pathway analysis, gene expression values for the significantly modulated genes were imported into the Ingenuity Pathway Analysis (IPA) software to identify canonical pathways associated with genes from the Ingenuity Pathways Analysis library.[24] The genes associated with a canonical pathway were measured in two ways: 1) Ratio of the number of

genes from the data set that map to the pathway is displayed. The ratio provides the percentage of genes in the dataset that were part of a defined list of genes associated with a particular pathway. 2) Fisher's exact test was used to calculate a p-value, which expresses the probability that the association between the genes in the dataset and the canonical pathway can be explained by chance alone; highly significant p-values support an alternate hypothesis that suggests that the interaction is not due to random chance.

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622 **Figure Legends**

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624 Figure 1: Venn diagram depicting the number of transcripts that were differentially expressed at
625 day 2, day 7, and day 14 post-immunization. The common and unique transcripts shown are
626 indicative of those which were statistically significant (FDR-corrected Step-up p-value ≤ 0.1) as
627 well meeting a minimum criteria of a twofold change in gene expression (either up or down)
628 over baseline levels of expression.

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631 Figure 2: Principal Component Analysis (PCA) Mapping of TC-83 *in vivo* vaccination
632 microarray data. PCA, as a visual representation of the experimental conditions which elicit the
633 greatest variability in the data, illustrated that the greatest differences in the data were due to the
634 effect of the treatment, with the samples from vaccinees immunized with TC-83 showing the
635 greatest diversity of gene expression. The second greatest factor which separates the data are the
636 changes due to or that occur over time following vaccination. The remaining factor which
637 describes the further diversification of data are the changes due to differences which are
638 introduced by the intersection of time and treatment.

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640 Figure 1:
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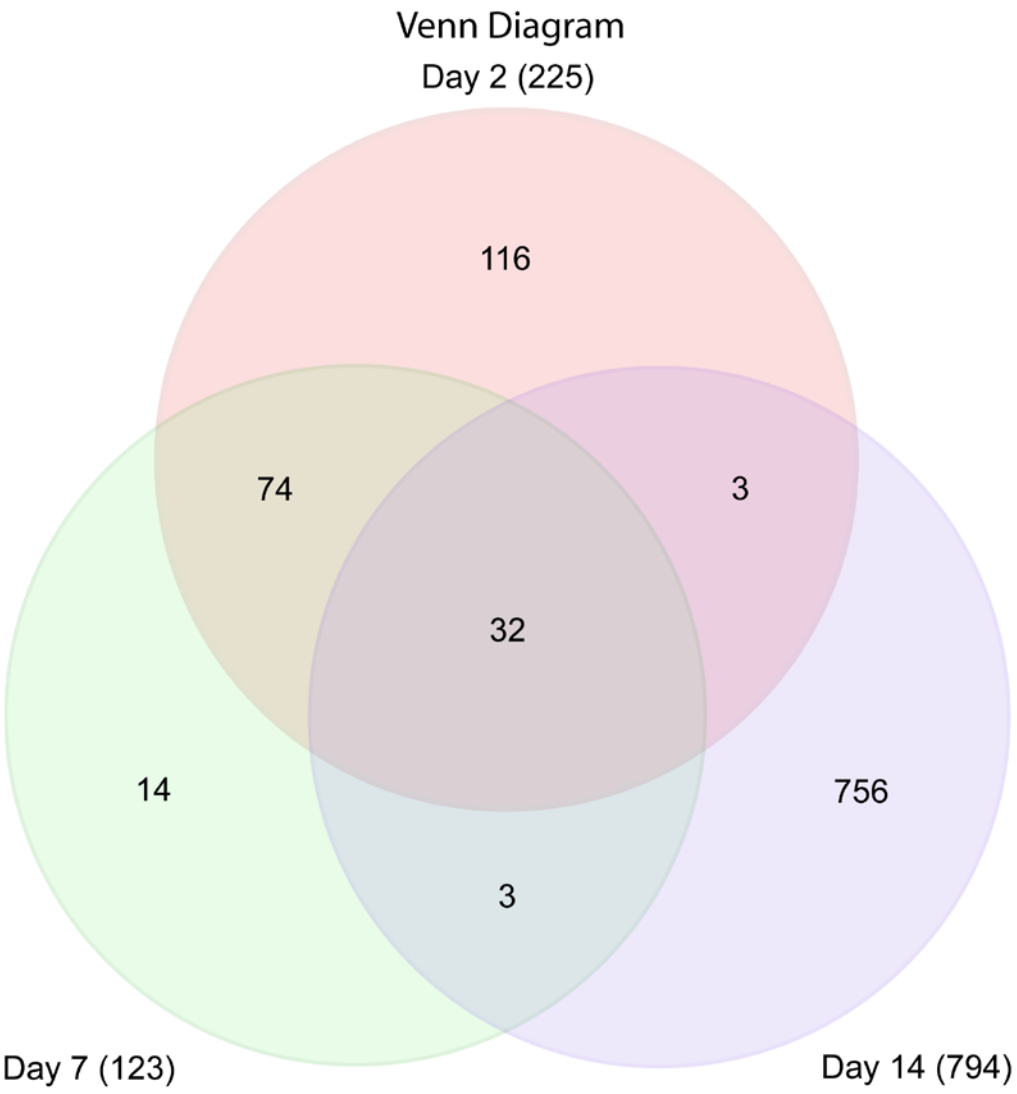

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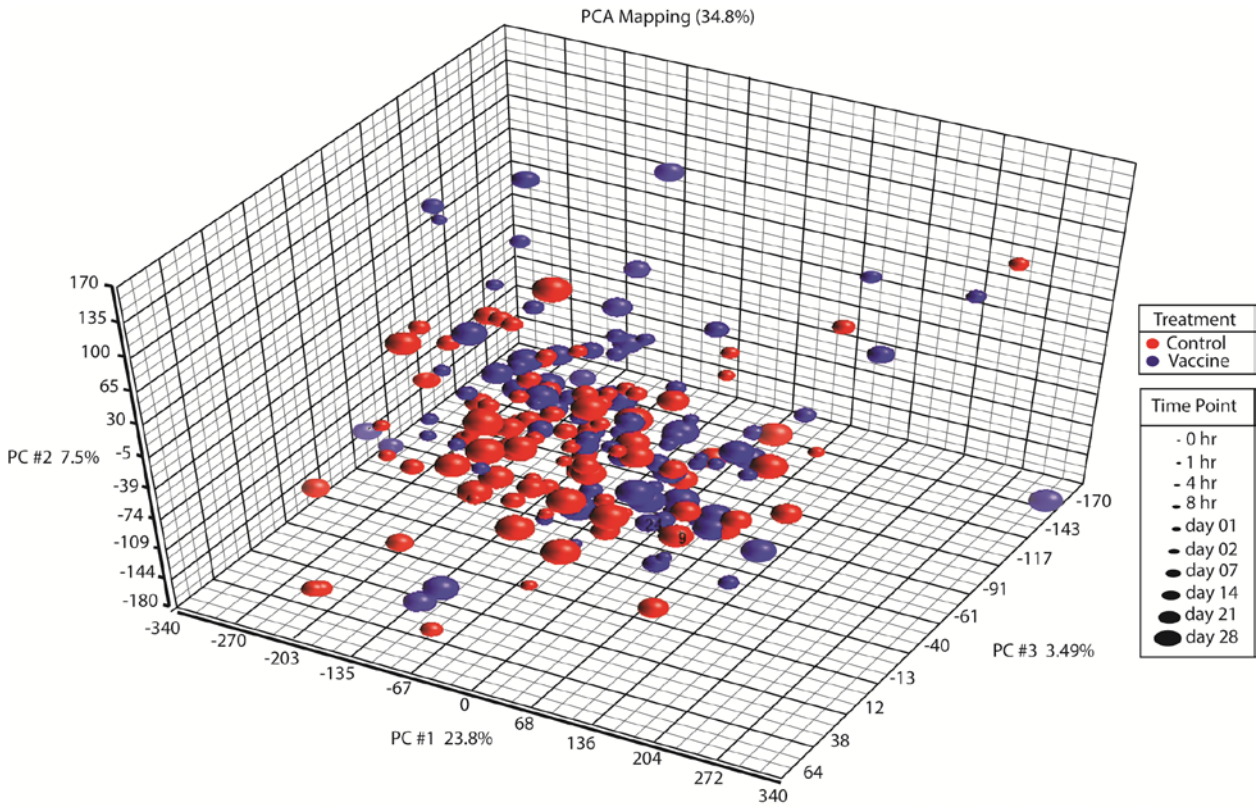
646 Figure 2: PCA mapping for *in vivo* experiments



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650 Table 1: FDR Report
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FDR Report		
Significance Level: 0.1; Total number of p-values: 54675		
Method: Step Up		
Variable Name	**Cutoff Value**	**# of Significant p-values**
p-value(Time Point)	1.49E-02	8,128
p-value(Treatment)	1.10E-05	6
p-value(Time Point * Treatment)	1.84E-02	10,055
p-value(0 h * Vaccine vs. 0 h * Control)	1.83E-06	0
p-value(1 h * Vaccine vs. 1 h * Control)	1.83E-06	0
p-value(4 h * Vaccine vs. 4 h * Control)	1.83E-06	0
p-value(8 h * Vaccine vs. 8 h * Control)	1.83E-06	0
p-value(day 1 * Vaccine vs. day 1 * Control)	1.83E-06	0
p-value(day 2 * Vaccine vs. day 2 * Control)	6.42E-03	3,511
p-value(day 7 * Vaccine vs. day 7 * Control)	7.75E-04	424
p-value(day 14 * Vaccine vs. day 14 * Control)	3.90E-02	21,343
p-value(day 21 * Vaccine vs. day 21 * Control)	1.83E-06	0
p-value(day 28 * Vaccine vs. day 28 * Control)	1.83E-06	0

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655 Table 2: Overview of Pathway Analysis Summary.
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Summary of IPA Analysis			
TOP BIO FUNCTIONS	**VEE Day 2**	**VEE Day 7**	**VEE Day 14**
Diseases and Disorders	Antimicrobial Response	Organismal Injury and Abnormalities	Immunological Disease
	Inflammatory Response	Antimicrobial Response	Hematological Disease
	Organismal Injury and Abnormalities	Inflammatory Response	Cancer
	Infection Mechanism	Infection Mechanism	Reproductive System Disease
	Infectious Disease	Genetic Disorder	Genetic Disorder
Molecular & Cellular Function	Cellular Movement	Post-Translational Modification	Nucleic Acid Metabolism
	Cellular Development	Protein Folding	Cell-to-Cell Signaling and Interaction
	Cell-to-Cell Signaling and Interaction	Cellular Development	Cellular Compromise
	Post-Translational Modification	Lipid Metabolism	Gene Expression
	Protein Folding	Molecular Transport	Molecular Transport
Physiology System Development & Function	Hematological System Development and Function	Endocrine System Development and Function	Tissue Development
	Immune Cell Trafficking	Hematological System Development and Function	Tumor Morphology
	Tissue Development	Hematopoiesis	Immune Cell Trafficking
	Skeletal and Muscular System Development and Function	Skeletal and Muscular System Development and Function	Nervous System Development and Function
	Hematopoiesis	Immune Cell Trafficking	Organ Morphology
TOP CANONICAL PATHWAYS	Interferon Signaling	Interferon Signaling	Oxidative Phosphorylation
	Activation of IRF by Cytosolic Pattern Recognition Receptors	Activation of IRF by Cytosolic Pattern Recognition Receptors	Protein Ubiquitination Pathway
	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	Regulation of eIF4 and p70S6K Signaling
	Role of RIG1-like Receptors in Antiviral Innate Immunity	Pathogenesis of Multiple Sclerosis	RAN Signaling
	IL-6 Signaling	Role of RIG1-like Receptors in Antiviral Innate Immunity	T Cell Receptor Signaling
Top Molecules - UP	RSAD2, IFI44L, IFIT1, AMPK2, ISG15, LAMP3, IFI44, HERC5, MX1, OAS3 (includes EG:4940)	IFI27, RSAD2, IFI44L, IFI44, ISG15, CMPK2, IFIT1, OAS3 (includes EG:4940), HERC5, OAS1	IFI27, IGJ, IGL@, IFI44, IGHM, IFI44L, RSAD2, TNFRSF17, TXNDC5, IGHA1
Top Molecules - DOWN	FCER1A, IL8, ITM2A, SGK1, GRAMD1C, IRS2, CLC, THBD, IGF1R, FAM101B	PI3, TUBB2A, EPB42, SLC4A1, SNCA, IGF1R, MARCH8, CPA3, FAM101B, CCR3	PI3, EPB42, TNS1, SLC4A1, TUBB2A, SELENBP1, SNCA, GMPR, KRT1, BLVRB,

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660 Table 3: Top Canonical Pathways in response to Live-Attenuated VEEV (TC-83) Vaccination.

Top Canonical Pathways							
Pathway	**Molecules**	**Day 2**		**Day 7**		**Day 14**	
Interferon Signaling		Fold Change	p-value*	Fold Change	p-value*	Fold Change	p-value*
	IFI35	**3.86**	**2.94E−14**	**2.84**	**9.43E−09**	1.33	1.01E−01
	IFIT1	**13.11**	**1.02E−06**	**10.95**	**2.53E−05**	**2.95**	**3.88E−02**
	IFIT3	**7.03**	**3.47E−08**	**7.34**	**2.57E−10**	1.88	**7.64E−02**
	IFITM1	**2.38**	**1.66E−07**	**2.35**	**1.30E−06**	1.61	**6.35E−03**
	MX1	**9.93**	**3.69E−09**	**6.89**	**4.17E−06**	1.71	1.73E−01
	OAS1	**7.18**	**2.32E−08**	**7.42**	**6.46E−08**	**2.71**	**7.01E−03**
	SOCS1	**2.43**	**5.90E−09**	1.58	**4.65E−02**	−1.20	1.19E−01
Activation of IRF by Cytosolic Factors	DDX58	**5.00**	**2.85E−09**	**2.79**	**1.86E−03**	1.85	**2.69E−02**
	DHX58	**2.00**	**9.01E−10**	1.50	**4.94E−03**	1.03	8.26E−01
	IFIH1	**4.50**	**7.41E−08**	**3.03**	**8.02E−04**	**2.36**	**4.12E−03**
	IFIT2	**5.54**	**1.67E−12**	**4.36**	**1.81E−04**	1.89	**6.96E−02**
	IRF7	**3.70**	**2.35E−09**	**3.68**	**1.85E−08**	1.40	1.27E−01
	ISG15	**11.39**	**3.77E−11**	**11.20**	**2.76E−10**	**2.66**	**1.07E−02**
	ZBP1	**3.94**	**1.32E−12**	**2.85**	**3.73E−09**	1.72	**3.11E−03**
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	DDX58	**5.00**	**2.85E−09**	**2.79**	**1.86E−03**	1.85	2.69E−02
	EIF2AK2	**3.27**	**8.67E−10**	**2.96**	**1.42E−07**	1.35	1.25E−01
	IFIH1	**4.50**	**7.41E−08**	**3.03**	**8.02E−04**	**2.36**	**4.12E−03**
	IRF7	**3.70**	**2.35E−09**	**3.68**	**1.85E−08**	1.40	1.27E−01
	OAS2	**5.35**	**4.31E−12**	**4.97**	**2.57E−10**	**2.24**	**1.42E−03**
	OAS3	**9.07**	**3.35E−12**	**8.20**	**1.58E−10**	**2.27**	**1.22E−02**
Role of RIG1-like Receptors in Antiviral Innate Immunity	DDX58	**5.00**	**2.85E−09**	**2.79**	**1.86E−03**	1.85	2.69E−02
	DDX60	**3.53**	**1.44E-04**	**4.43**	**1.18E-05**	**3.49**	**3.78E-04**
	DHX58	**2.00**	**9.01E−10**	**1.50**	**4.94E−03**	1.03	8.26E−01
	TLR7	1.58	**1.39E-06**	1.12	7.93E-01	-1.10	3.08E-01
Interleukin-6 Signaling	IL8	**−3.11**	**2.25E−02**	1.36	8.85E−01	1.74	1.62E−01
	IL1RN	**3.46**	**4.00E−09**	**2.28**	**1.20E−03**	−1.19	**5.68E−02**
	TNFAIP6	**3.87**	**2.39E−06**	**2.84**	**2.31E−03**	1.53	2.19E−01
Communication between Innate and Adaptive Immune	CXCL10	**3.79**	**1.31E−12**	**2.10**	**5.95E−04**	1.21	3.10E−01
	IL1RN	**3.46**	**4.00E−09**	**2.28**	**1.20E−03**	−1.19	**5.68E−02**
	TNFSF13B	**2.48**	**1.70E−05**	**1.96**	**1.27E−02**	1.35	2.47E−01
JAK/STAT Signaling	SOCS1	**2.43**	**5.90E−09**	1.58	**4.65E−02**	−1.20	1.19E−01
	STAT1	**2.67**	**1.59E−05**	**1.99**	**2.68E−02**	1.57	**1.89E−02**
	STAT2	**2.83**	**5.50E−09**	**2.19**	**1.10E−04**	1.46	**3.75E−02**
Oxidative Phosphorylation	ATP5J	-1.30	4.94E-01	1.34	8.34E-01	**2.20**	**9.53E-03**
	COX7B	-1.40	5.53E-01	1.60	8.10E-01	**2.20**	**6.43E-02**
	COX7A2	-1.27	5.13E-01	1.43	7.64E-01	**2.04**	**1.35E-02**
	COX6C	-1.16	7.86E-01	1.80	6.65E-01	**2.50**	**1.59E-02**
	UQCRB	-1.24	7.46E-01	1.67	8.08E-01	**2.32**	**7.13E-02**
	UQCRH	-1.13	7.88E-01	1.46	7.79E-01	**2.35**	**7.71E-03**
	PPA1	-1.06	8.71E-01	1.44	6.94E-01	**2.23**	**2.18E-03**
	NDUFA6	-1.25	5.26E-01	1.48	7.12E-01	**2.11**	**7.78E-03**
	UQCRQ	-1.18	7.41E-01	1.56	7.68E-01	**2.22**	**2.44E-02**
Protein Ubiquitination Pathway	PSMA3	1.17	7.02E−01	1.43	7.86E−01	**2.42**	**5.05E−03**
	UBR1	−1.27	5.23E−01	1.16	9.30E−01	**2.48**	**3.00E−03**
	USP1	−1.74	7.37E−02	1.06	9.70E−01	**2.32**	**8.32E−03**
	UBE3A	−1.54	1.91E−01	1.18	8.48E−01	**2.07**	**7.17E−03**
	USP53	−1.08	6.88E−01	1.21	9.10E−01	**2.28**	**9.92E−03**
	PSMC6	−1.72	3.37E−01	1.20	9.48E−01	**2.71**	**2.57E−02**
	USP47	−1.56	1.99E−01	1.21	8.97E−01	**2.11**	**8.24E−03**
	USP16	−1.64	1.67E−01	1.22	8.66E−01	**2.22**	**6.24E−03**
	PSMA4	1.10	8.61E−01	1.69	7.09E−01	**2.54**	**1.08E−02**
	HSP90AA1	−1.02	9.78E−01	1.51	7.94E−01	**2.91**	**4.74E−03**
	BIRC3	−1.50	2.52E−01	1.09	9.63E−01	**2.12**	**1.62E−02**
	BIRC2	−1.38	4.13E−01	1.08	9.70E−01	**2.22**	**5.80E−03**
ERK5 signaling	YWHAQ	−1.25	4.08E−01	1.20	8.27E−01	**2.27**	**4.00E−04**
	IL6ST	−1.43	2.98E−01	1.24	8.51E−01	**2.07**	**8.76E−03**
	RPS6KB1	−1.58	1.82E−01	1.09	9.15E−01	**2.11**	**7.34E−03**
	NRAS	−1.24	4.26E−01	1.21	7.60E−01	**2.08**	**1.65E−03**
	RRAS2	−1.39	2.32E−01	1.17	8.92E−01	**2.31**	**4.53E−04**
	ATF2	−1.48	2.86E−01	1.04	9.27E−01	**2.32**	**5.48E−03**
Natural Killer Cell Signaling	FYN	−1.45	1.65E−01	1.22	7.18E−01	**2.09**	**1.44E−03**
	PRKCI	−1.31	3.57E−01	1.26	7.35E−01	**2.04**	**3.45E−03**
	NRAS	−1.24	4.26E−01	1.21	7.60E−01	**2.08**	**1.65E−03**
	RRAS2	−1.39	2.32E−01	1.17	8.92E−01	**2.31**	**4.53E−04**
	PIK3C2A	−1.57	1.79E−01	1.23	6.55E−01	**2.03**	**9.01E−03**
	KLRK1	−1.11	7.89E−01	1.21	8.78E−01	**2.01**	**8.80E−03**
B Cell Development	IL7R	nd	nd	nd	nd	**2.13**	**4.13E−03**
	IGKC	−1.02	9.57E−01	1.04	9.79E−01	**2.44**	**6.13E−04**
	IGL@	1.30	**8.19E−02**	1.29	8.52E−01	**6.01**	**2.87E−12**
	IGHM	−1.48	2.48E−01	−1.28	8.48E−01	**4.99**	**7.60E−04**
T Cell Receptor Signaling	FYN	-1.45	1.65E-01	1.13	9.13E-01	**2.09**	**1.44E-03**
	CD28	-1.57	1.72E-01	1.05	9.79E-01	**2.57**	**9.73E-04**
	CAMK4	-1.86	**5.56E-02**	1.04	9.83E-01	**2.10**	**4.89E-03**
	RRAS2	-1.39	2.32E-01	1.17	8.92E-01	**2.31**	**4.53E-04**
	PI3KC2A	-1.57	1.79E-01	-1.03	9.88E-01	**2.03**	**9.01E-03**
	RASGRIP1	-1.48	1.70E-01	1.23	8.50E-01	**2.44**	**3.48E-04**
	CD3D	-1.14	7.40E-01	1.50	7.02E-01	**2.24**	**4.84E-03**
	ITK	-1.44	1.83E-01	1.15	9.04E-01	**2.12**	**1.44E-03**
*p-values shown are the FDR-corrected Step up values.							
nd = not detected							

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Table 4: Identification of Biomarkers following Vaccination with Live-Attenuated (TC-83) VEEV

Table 4: Top Biomarkers							
Gene symbol	**Gene Name**	**Cellular Location**	**Fold Change**	**Day 2**	**Day 7**	**Day 14**	**Common**
GBP4	guanylate binding protein 4	Cytoplasm	3.5	X			
MT1X	metallothionein 1X	unknown	3.2	X			
ANKRD22	ankyrin repeat domain 22	Nucleus	3.2	X			
CCL2	chemokine (C-C motif) ligand 2	Extracellular Space	3.1	X			
BST2	bone marrow stromal cell antigen 2	Plasma Membrane	3.0	X			
LIPA	lipase A, lysosomal acid, cholesterol esterase	Cytoplasm	2.8	X			
TRIM14	tripartite motif containing 14	Cytoplasm	2.3		X		
CCR1	chemokine (C-C motif) receptor 1	Plasma Membrane	2.2		X		
SMA5	glucuronidase, beta pseudogene	unknown	2.2		X		
PPM1K	protein phosphatase, Mg2+/Mn2+ dependent, 1K	Cytoplasm	2.1		X		
SHISA5	shisa homolog 5 (Xenopus laevis)	Nucleus	2.0		X		
C18orf49	chromosome 18 open reading frame 49	unknown	2.0		X		
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	Extracellular Space	6.8			X	
IGL@	immunoglobulin lambda locus	Nucleus	6.0			X	
IGHM	immunoglobulin heavy constant mu	Plasma Membrane	5.0			X	
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	Plasma Membrane	4.1			X	
TXNDC5	thioredoxin domain containing 5 (endoplasmic reticulum)	Cytoplasm	4.0			X	
NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	Cytoplasm	3.4			X	
CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	Cytoplasm	3.5 to 11.9				X
RSAD2	radical S-adenosyl methionine domain containing 2	Cytoplasm	4.2 to 29.2				x
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	unknown	3.5 to 4.4				X
EPSTI1	epithelial stromal interaction 1 (breast)	unknown	4.1 to 8.2				X
HERC5	hect domain and RLD 5	Cytoplasm	2.2 to 10.2				X
LY6E	lymphocyte antigen 6 complex, locus E	Plasma Membrane	2.4 to 6.5				X
RTP4	receptor (chemosensory) transporter protein 4	Plasma Membrane	2.2 to 4.3				X
XAF1	XIAP associated factor 1	Nucleus	2.3 to 6.2				X

669 Table 5: HLA phenotype and post vaccination titer of Study Volunteers

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Treatment	HLA-DRB1 Phenotype	HLA-DQB1 Phenotype	Day 28 Post-TC-83 Vaccination Titer
Control 1	0401/1501	0302/0602	**<10**
Control 2	0401/0701	0301/0319 / 0202	**<10**
Control 3	0301/0701	0201/0202	**<10**
Control 4	0701/1302	0202/0302	**<10**
Control 5	0101/0701	0501/0303	**<10**
Control 6	0301/1602	0201/0502	**<10**
Control 7	0401/0701	0302/0202	**<10**
Control 8	0101/0701	0501/0303	**<10**
Control 9	0401/0701	0301/0319 / 0202	**<10**
Control 10	0302/1503	0402/0602	**<10**
Vaccinee 1	1101/1302	0301/0319 / 0604/0634	**<10***
Vaccinee 2	0402/0701	0302/0202	**20**
Vaccinee 3	0301/0401	0202/0602	**40**
Vaccinee 4	0402/0701	0302/0202	**80**
Vaccinee 5	0801/1501	0402/0602	**160**
Vaccinee 6	1101/1302	0301/0319 / 0609	**160**
Vaccinee 7	0401/0701	0202/0302	**320**
Vaccinee 8	0701/1501	0202/0602	**320**
Vaccinee 9	0701/1401	0303/0503	**1280**
Vaccinee 10	1501	0602	**1280**
* Titer repeated at Day 56; Subject confirmed as NonResponder			

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672 *Day 56 titer indicates that Vaccinee 1 was a Nonresponder.
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675 Supplemental Data Table 1: FDR Report for ANOVA analysis with the added variable of

676 vaccine response

FDR Report		
Significance Level: 0.1; Total number of p-values: 54675		
Method: Step Up		
Variable Name	**Cutoff Value**	**# of Significant p-values**
p-value(Time Point)	1.24E-02	6795
p-value(Treatment)	3.66E-06	2
p-value(Vaccine Response(Treatment))	1.83E-06	0
p-value(Time Point * Treatment)	1.72E-03	939
p-value(Time Point * Vaccine Response(Treatment))	3.66E-06	2
p-value(Vaccine * 0 hr * High and Control * 0 hr * Control vs. Vaccine * 0 hr * Low and Control * 0 hr * Control)	1.83E-06	0
p-value(Vaccine * 1 hr * High and Control * 1 hr * Control vs. Vaccine * 1 hr * Low and Control * 1 hr * Control)	1.83E-06	**1**
p-value(Vaccine * 4 hr * High and Control * 4 hr * Control vs. Vaccine * 4 hr * Low and Control * 4 hr * Control)	1.83E-06	0
p-value(Vaccine * 8 hr * High and Control * 8 hr * Control vs. Vaccine * 8 hr * Low and Control * 8 hr * Control)	1.28E-05	**7**
p-value(Vaccine * day 01 * High and Control * day 01 * Control vs. Vaccine * day 01 * Low and Control * day 01 * Control)	1.83E-06	0
p-value(Vaccine * day 02 * High and Control * day 02 * Control vs. Vaccine * day 02 * Low and Control * day 02 * Control)	1.83E-06	**1**
p-value(Vaccine * day 07 * High and Control * day 07 * Control vs. Vaccine * day 07 * Low and Control * day 07 * Control)	1.04E-04	**57**
p-value(Vaccine * day 14 * High and Control * day 14 * Control vs. Vaccine * day 14 * Low and Control * day 14 * Control)	2.38E-05	**13**
p-value(Vaccine * day 21 * High and Control * day 21 * Control vs. Vaccine * day 21 * Low and Control * day 21 * Control)	1.83E-06	0
p-value(Vaccine * day 28 * High and Control * day 28 * Control vs. Vaccine * day 28 * Low and Control * day 28 * Control)	1.83E-06	0

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679 Supplemental Data Table 2: 5-Way ANOVA_Time-Treatment vs Titer (See attached Excel file,
680 file is too large to embed).
681 Supplemental Data Table 3: MicroRNA Target Filter List for VEEV Vax Days 2-7-14
682 (Attached).